TRIVALENT THROMBIN INHIBITOR

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a thrombin inhibitor, a pharmaceutical composition comprising same and a method for the treatment or prevention of vascular disease using the inhibitor of the present invention.

(b) Description of Prior Art

Thrombin plays a central role in the coagulation cascade of higher animals. The primary function of thrombin is to activate fibrinogen to fibrin and generate an insoluble fibrin clot. It also serves regulatory functions in coagulopathy by activating several participating cofactors and proteases such as factor V, factor VIII, factor XIII and protein C. In a pathologic state, thrombin promotes coagulopathy, activates platelets and causes secretion of granular substances that exacerbate the condition. Thrombin's interaction with endothelial cells, smooth muscle cells, fibroblasts, and monocytes/macrophages contribute further to the inflammatory process in thrombolytic events. Heart attack is one of the most important kind of cardiovascular diseases. An acute blockage of a coronary artery by a thrombus causes a myocardial infarction. If a large artery, which nourishes a large part of the heart, is blocked, the attack is more likely to be fatal. In fact, 40% of death in North America is attributes to cardiovascular diseases. The chances of recovery are good if the blockage occurs in one of the smaller coronary arteries. In its early stages, the condition may be alleviated with thrombolytic therapy. However, typical thrombolysis with tissue plasminogen activator, urokinase or streptokinase is problematic. These enzymes activate

plasminogen to plasmin, which in turn lyses fibrin present in a coronary thrombus, thus restoring the blood flow to the heart muscle. Acute thrombotic reocclusion often occurs after initial successful thrombolysis using these agents. Although the mechanism of reocclusion has not been clearly elucidated, thrombus-bound thrombin may contribute to this problem. In fact, thrombus-bound thrombin, which is still active, has been suggested to contribute to rethrombosis after thrombolytic therapy (Agnelli, G., et al., *J. Thrombosis and Haemostasis* **66**, 592-597, 1991). Potent and specific agents that neutralize thrombus-bound thrombin would be desirable.

Thrombin is a member of the trypsin family of serine proteases. In addition to the catalytic triad (Asp 102, His 57 and Ser 195) a feature common to the active site of all serine proteases, Asp 189 in the primary substrate binding site (S1) of the trypsin family plays an important role in the recognition and binding of substrates and inhibitors.

Several approaches have been taken to design anticoagulant agents. 1) Since one of the major component of thrombus is aggregated platelet-fibrinogen, drugs which prevent the aggregation between platelet and fibrinogen have been designed. A sequence of Arg-Gly-Asp in fibrinogen is responsible to interact with activated platelet so that many peptide or non-peptide based drugs which mimic the tripeptide structure have been developed. Antibodies which block the platelet fibrinogen receptor, Gp IIb/IIIa, have also been developed. 2) Tissue factor pathway inhibitor, which inhibits tissue factor and factor VIIa complex, blocks the early stage of coagulation cascade. 3) Protein C is a natural anticoagulant and inactivates factors Va and VIIIa. 4) Currently available drugs

which may not be optimized and use in a combination of the existing drugs has been studied. 5) Thrombin plays a central role in coagulation, thrombosis and platelet activation. The direct inhibition of thrombin activity has advantages of independence to co-factors, efficacy to clot-bound thrombin, less patient-to-patient variability, and low risk of bleeding.

There is no satisfactory drug in the market. As a result, a natural anti-coagulant, heparin, which has some side effects and low efficacy (but low cost), is still used in the hospital.

Heparin inhibits thrombin through a mechanism requiring a heparin-antithrombin III complex. Heparin is known to be poorly accessible to thrombus-bound thrombin. Furthermore, heparin often causes bleeding when used therapeutically and is unable to prevent the occlusive complications in atherosclerotic vascular diseases or reocclusion following successful thrombolysis.

Another agent known to be effective for the inhibition of thrombus-bound thrombin is hirudin. Hirudin is produced by the salivary glands of the European medicinal leech Hirudo medicinalis and is a small protein of 65 amino acid residues. It has several potential advantages over other antithrombotics. It is the most potent and specific thrombin inhibitor known having a K_i value of 2.2×10^{-14} M. Hirudin blocks the active site (AS) and the fibrinogen recognition exosite (FRE) of thrombin simultaneously. Hirudin also inhibits thrombus-bound thrombin as well as circulating thrombin and it has a long half-life of 30-60 minutes when given intravenously or subcutaneously, depending on the species. Hirudin has very weak antigenicity, and it

has no reported acute side effect following intravenous or subcutaneous administration.

Synthetic thrombin inhibitors based on the hirudin sequence offer an advantage over native hirudin. They mimic the distinctive mechanism of hirudin and are more readily available through chemical synthesis. The crystal structure of the human α-thrombin/hirudin complex reveals that hirudin interacts with the enzyme through an active site inhibitor domain (hirudin$^{1-48}$), a FRE inhibitor segment (hirudin$^{55-65}$), and a linker segment (hirudin$^{49-54}$) which connects these binding components.

The bulky active site inhibitor segment, hirudin $^{1-48}$, is sufficiently large and serves to obstruct the enzyme surface. This action has been shown to be simulated when hirudin $^{1-48}$ is replaced by a small active site inhibitor segment, (D-Phe)-Pro-Arg-Pro, with some loss in inhibitory potency (Maraganore, J.M., et al., *Biochemistry* **29**, 7095-7101, 1990; DiMaio, J., et al., *J.Biol.Chem* **265**, 21698-21703, 1990; and Bourdon, P., et al., *FEBS Lett.* **294**, 163-166, 1991).

Investigators have focused on the use of (D-Phe)-Pro-Arg-Pro or its analog in the design of active site inhibitors. The crystal structure of (D-Phe)-Pro-Arg chloromethylketone (PPACK)-thrombin suggested that the (D-Phe)-Pro-Arg-Pro in bivalent inhibitors bind to the thrombin active site in a substrate binding mode, wherein Arg-X is the scissile peptide bond. The active site inhibitor segment, (D-Phe)-Pro-Arg-Pro, of the bivalent inhibitors is known to be hydrolyzed slowly by thrombin (DiMaio, J., et al., ***Supra***; Witting, J.I., et al., *BioChem. J.* **287**, 663-664, 1992). The amino acids (D-Phe)-Pro-Arg comprised in the substrate type inhibitor (D-Phe)-Pro-

Arg-Pro bind to the S3, S2 and S1 subsites of thrombin, respectively.

Hirulog-8™ is a bivalent thrombin inhibitor composed of the substrate type inhibitor (D-Phe)-Pro-Arg-Pro, and the native sequence of the hirudin exosite segment 52-65 both linked through a suitable linker (Maraganore et al. US Patent No. 5,196,404). Since the structure of the active site inhibitor segment is very similar to the structure of PPAC, the interactions of the substrate type active site inhibitor with thrombin is reasonably assumed to be the same as the interactions between the active site of PPAC and thrombin. In addition, it has been shown that the portion (D-Phe)-Pro-Arg-CO can be used in a bivalent thrombin inhibitor (DiMaio et al. International publication WO 91/19734). The scissile position in a substrate is a position that is recognised by the enzyme and where the hydrolysis takes place. It is therefore advantageous to eliminate or to modify the scissile position in order to give to more resistance to enzyme degradation. The synthesis of such inhibitors is difficult, cumbersome, uses dangerous chemicals and affords low yields of the desired compounds. There is therefore a need for other thrombin inhibitors that would combine high inhibiting activity, enzyme resistance and affordable synthesis.

Besides substrate-type inhibitors, nonsubstrate type inhibitors could be designed to block the active site of thrombin without being cleaved. Examples of these may be derived from arginine and benzamidine to give, for example, (2R,4R)-4-methyl-1-[N^{α}-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulphonyl)-L-arginyl]-2-piperidine carboxylic acid (MD-805), N^{α}-(4-toluene-sulphonyl)-D,L-amidinophenylalanyl-piperidine (TAPAP), and Nα-(2-naphthyl-sulphonyl-glycyl)-D-L,p-amidino-

phenylalanyl-piperidine (NAPAP). These active-site directed synthetic inhibitors have a short half-life of less than several minutes in the circulation. This activity is not of sufficient duration to be effective against the continuous production of thrombin by the patient or against the effect of liberated thrombus bound-thrombin. The characteristic sequence of these compounds starting from the N-terminus is an aromatic group, arginyl or benzamidyl, and piperidide or its analogs. In contrast to hirudin-based sequences, these moieties would be expected to occupy the S3, S1 and S2 subsites of the thrombin active site, respectively.

It has been previously reported that the combination of dansyl or dansyl analogues, arginine or benzamidine, and pipecolic acid attaches to the thrombin active site. But it has been shown that such activity is weak and not pharmacologically useful (James C. Powers and Chih-Min Kam, *Thrombin: Structure and Function*, Chapter 4, (1992), Lawrence J.Berliner, Plenum Press, New York).

A short sequence of a hirudin type inhibitor having non-substrate type active site inhibitor segment and the fibrinogen-recognition exosite inhibitor segment, has also been previously reported. (Tsuda, Y., et al., *Biochemistry* 33: 14443-14451, 1994).

It would be highly desirable to be provided with a shortened thrombin inhibitor of the hirudin type. Such a shorter sequence would be easier to synthesize and cheaper to produce. It would have a linear sequence less subject to enzymatic degradation and would be more stable when bound to thrombin.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a new trivalent thrombin inhibitor which as a high

affinity for thrombin and which is more stable when such inhibitor is bound to thrombin.

In accordance with the present invention there is provided a new thrombin inhibitor of formula (I) or a pharmaceutically acceptable salt thereof:

AS-Z-P (I)

wherein

AS represents an S subsite blocking segment;

P represents a fibrinogen recognition exosite blocking segment; and

Z represents a S' subsite blocking segment which links AS and P, said S' subsite blocking segment having the following sequence:

Xaa-Gly-Yaa-Gly-βAla

wherein Xaa is a residue selected from the group of residue consisting of glycine, L-alanine, D-alanine, 2-aminoisobutyric acid, L-α-aminobutyric acid, D-α-aminobutyric acid, L-norvaline, D-norvaline, L-norleucine, D-norleucine, L-cysteine, L-penicillamine, D-penicillamine, L-methionine, D-methionine, L-valine, D-valine, L-tert-butylglycine, D-tert-butylglycine, L-isoleucine, D-isoleucine, L-leucine, D-leucine, cyclohexylglycine, L-β-cyclohexylalanine, D-β-cyclohexylalanine, L-phenylglycine, D-phenylglycine, L-phenylalanine, D-phenylalanine, L-homophenylalanine, D-homophenylalanine, L-histidine, D-histidine, L-tryptophan, D-tryptophan, L-β-(2-thienyl)-alanine, and D-β-(2-thienyl)-alanine;

Yaa is selected from the group of residue consisting of glycine, L-alanine, D-alanine, 2-aminoisobutyric acid, L-α-aminobutyric acid, D-α-aminobutyric acid, L-norvaline, D-norvaline, L-norleucine, D-norleucine, L-cysteine, L-penicillamine, D-penicillamine, L-methionine, D-methionine, L-valine, D-valine, L-tert-butylglycine, D-tert-butylglycine, L-isoleucine,

D-isoleucine, L-leucine, D-leucine, cyclohexylglycine, L-β-cyclohexylalanine, D-β-cyclohexylalanine, L-phenylglycine, D-phenylglycine, L-phenylalanine, D-phenylalanine, homophenylalanine, histidine, L-tryptophan, D-tryptophan, L-β-(2-thienyl)-alanine, and D-β-(2-thienyl)-alanine.

DETAILED DESCRIPTION OF THE INVENTION

Hirudin from medicinal leech is the most potent thrombin inhibitor. The high affinity of hirudin comes from the simultaneous binding to the active site and to the fibrinogen recognition exosite of thrombin. Synthetic thrombin inhibitors have been designed to mimic the binding mode of hirudin and composed of the active site blocking segment, the fibrinogen recognition exosite blocking segment, and the linker connecting these blocking segments. Surprisingly, it has been found that two residues, identified as P1' and P3', of the linker can form nonpolar interactions with thrombin. In accordance with the present invention, the linker, besides being a spacer, can be a binding segment to thrombin S' subsites. In this invention, the P1' and P3' residues were designed to optimize the interactions with thrombin.

In accordance with the present invention, there is therefore provided a trivalent thrombin inhibitor comprising a S subsite blocking segment, which is connected to the S' subsite blocking segment, which is connected to the fibrinogen recognition exosite blocking segment. In this invention, the design of the S' subsite blocking segment improved the affinity of the inhibitors by 250—300-fold which is significant and valuable commercially.

In accordance with one embodiment of the present invention, the trivalent thrombin inhibitors may be described by formula (I) which comprises an active site blocking segment (AS) linked to a S' sites blocking segment (Z) which serves as a linker and a fibrinogen recognition exosite blocking segment (P) linked to that linker:

AS-Z-P (I)

The AS blocking segment preferably has the following sequence:

Bbs-Arg-(D-Pip),

wherein Bbs and D-Pip represent 4-tert-butylbenzenesulfonyl and D-pipecolic acid, respectively.

The P segment preferably has the following sequences:

Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH, SEQ ID NO:1

wherein Cha represent β-cyclohexyl-alanine.

The z segment preferably has the following sequence:

Xaa-Gly-Yaa-Gly-βAla. SEQ ID NO:2

In accordance with a preferred embodiment of the present invention, when Xaa is a Glycine residue, Yaa is selected from the group of residue consisting of glycine, L-alanine, D-alanine, 2-aminoisobutyric acid, L-α-aminobutyric acid, D-α-aminobutyric acid, L-norvaline, D-norvaline, L-norleucine, D-norleucine, L-cysteine, L-penicillamine, D-penicillamine, L-methionine, D-methionine, L-valine, D-valine, L-tert-butylglycine, D-tert-butylglycine, L-isoleucine, D-isoleucine, L-leucine, D-leucine, cyclohexylglycine, L-β-cyclohexylalanine, D-β-cyclohexylalanine, L-phenyl-

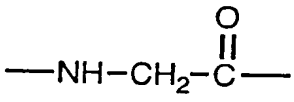
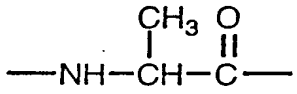
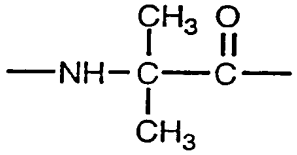
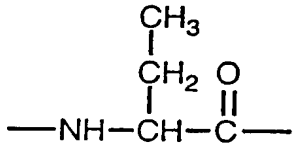
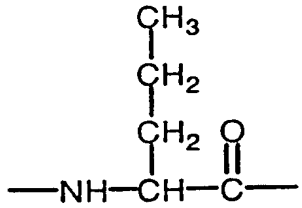
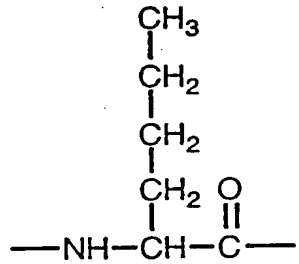
glycine, D-phenylglycine, L-phenylalanine, D-phenylalanine, homophenylalanine, histidine, L-tryptophan, D-tryptophan, L-β-(2-thienyl)-alanine, and D-β-(2-thienyl)-alanine.

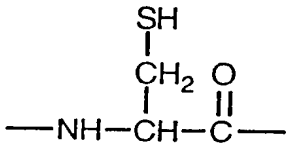
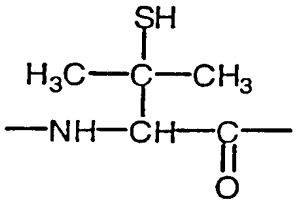
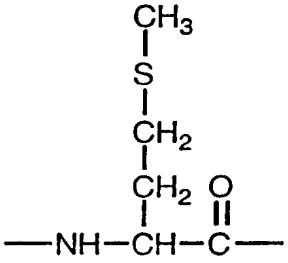
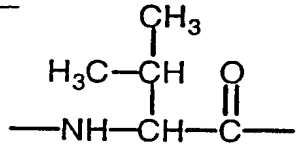
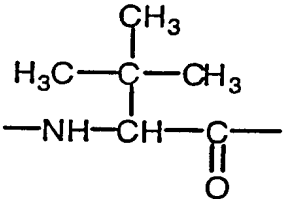
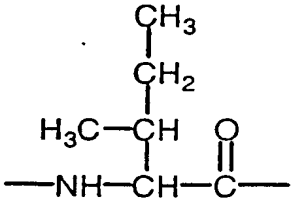
When Yaa is a glycine residue, Xaa is a residue selected from the group of residue consisting of glycine, L-alanine, D-alanine, 2-aminoisobutyric acid, L-α-aminobutyric acid, D-α-aminobutyric acid, L-norvaline, D-norvaline, L-norleucine, D-norleucine, L-cysteine, L-penicillamine, D-penicillamine, L-methionine, D-methionine, L-valine, D-valine, L-tert-butylglycine, D-tert-butylglycine, L-isoleucine, D-isoleucine, L-leucine, D-leucine, cyclohexylglycine, L-β-cyclohexylalanine, D-β-cyclohexylalanine, L-phenylglycine, D-phenylglycine, L-phenylalanine, D-phenylalanine, L-homophenylalanine, D-homophenyl-alanine, L-histidine, D-histidine, L-tryptophan, D-tryptophan, L-β-(2-thienyl)-alanine, and D-β-(2-thienyl)-alanine.

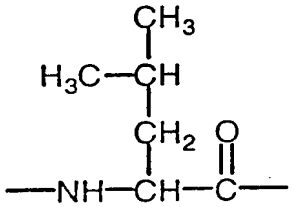
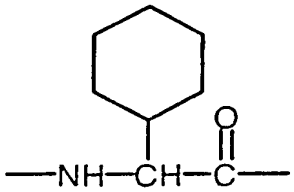
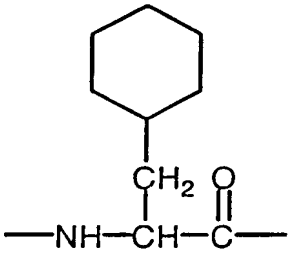
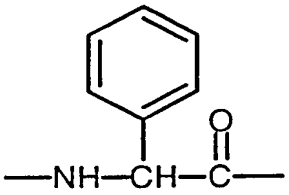
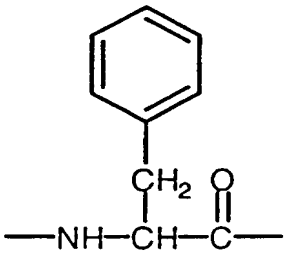
It should be noted that a person skilled in the art could substitute Gly, Gly and β-Ala in the Z segment with other amino acids, or could substitute both Xaa and Yaa simultaneously.

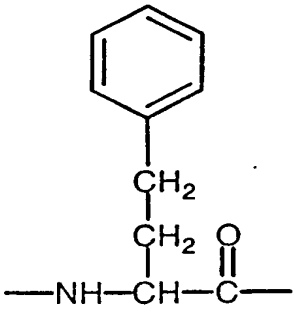
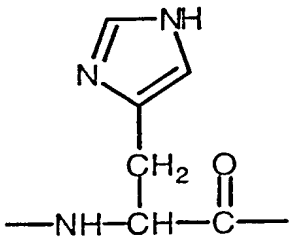
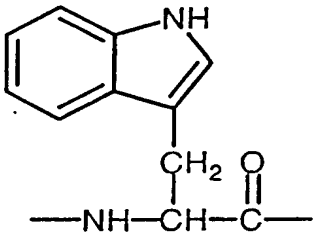
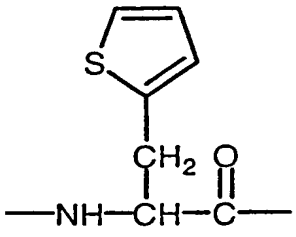
Table 1 below list the preferred Xaa and Yaa residues in accordance with the present invention, together with the Ki value, when available, of the inhibitor obtained. The following abbreviations have been used: αAib, 2-aminoisobutyric acid; αAbu, α-aminobutyric acid; Bbs, 4-tert-butylbenzenesulfonyl; Cha, β-cyclohexyl-alanine; Chg, cyclohexyl-glycine; Hph, homophenylalanine; Nva, norvaline; Nle, norleucine, Pen, Penicillamine; Phg, phenylglycine; Tbg, tert-butylglycine; and Thi, β-(2-thienyl)-alanine.

TABLE 1

Xaa or Yaa formula	P1' residue, Xaa Yaa = Gly	P3' residue, Yaa Xaa = Gly
	Xaa = Gly K_i=24 ± 5 pM	Yaa = Gly K_i=24 ± 5 pM
	Xaa = Ala K_i=1.2 ± 0.4 pM Xaa = D-Ala K_i=4.2 ± 0.5 pM	Yaa = Ala K_i=8.7 ± 0.2 pM Yaa = D-Ala K_i=0.96 ± 0.03pM
	Xaa = αAib K_i=2.4 ± 0.5pM	Yaa = αAib K_i=1.4 ± 0.3 pM
	Xaa = αAbu K_i=0.63 ± 0.05 pM Xaa = D-αAbu K_i=4.25 ± 0.4 pM	Yaa = αAbu K_i=7.4 ± 0.3 pM Yaa = D-αAbu K_i=0.77 ± 0.03 pM
	Xaa = Nva K_i=0.24 ± 0.05 pM Xaa = D-Nva K_i=5.1 ± 0.4 pM	Yaa = Nva K_i=9.2 ± 0.4 pM Yaa = D-Nva K_i=0.88 ± 0.04 pM
	Xaa = Nle K_i=0.082 ± 0.006 pM Xaa = D-Nle K_i=5.3 ± 0.3 pM	Yaa = Nle K_i=8.9 ± 0.5 pM Yaa = D-Nle K_i=0.68 ± 0.04 pM

	Xaa = Cys K_i=1.2 ± 0.3 pM	Yaa = Cys K_i=8.9 ± 0.5 pM
	Xaa = Pen K_i=1.5 ± 0.4 pM Xaa = D-Pen K_i=6.8 ± 0.5 pM	Yaa = Pen K_i+5.6 ± 0.5 pM Yaa = D-Pen K_i=1.5 ± 0.5 pM
	Xaa = Met K_i=0.11 ± 0.03 pM Xaa = D-Met K_i=4.8 ± 0.3 pM	Yaa = Met K_i=10.4 ± 0.5 pM Yaa = D-Met K_i=1.5 ± 0.5 pM
	Xaa = Val K_i=0.84 ± 0.05 pM Xaa = D-Val K_i=3.7 ± 0.4 pM	Yaa = Val K_i=1.2 ± 0.5 pM Yaa = D-Val K_i=0.62 ± 0.02 pM
	Xaa = Tbg K_i=1.1 ± 0.3 pM Xaa = D-Tbg K_i=5.8 ± 0.4 pM	Yaa = Tbg K_i=4.3 ± 0.5 pM Yaa = D-Tbg K_i=0.44 ± 0.04 pM
	Xaa = Ile K_i=0.14 ± 0.04 pM Xaa = D-Ile K_i=4.3 ± 0.3 pM	Yaa = Ile K_i=1.9 ± 0.3 pM Yaa = D-Ile K_i=0.35 ± 0.5 pM

	Xaa = Leu K_i=0.12 ± 0.04 pM Xaa = D-Leu K_i=2.6 ± 0.5 pM	Yaa = Leu K_i=2.2 ± 0.5 pM Yaa = D-Leu K_i=0.62 ± 0.03 pM
	Xaa = Chg K_i=0.35 ± 0.5 pM	Yaa = Chg K_i=3.2 ± 0.4 pM
	Xaa = Cha K_i=0.12 ± 0.04 pM Xaa = D-Cha K_i=7.2 ± 0.3 pM	Yaa = Cha K_i=9.6 ± 0.5 pM Yaa = D-Cha K_i=1.5 ± 0.5 pM
	Xaa = Phg K_i=3.1 ± 0.4 pM Xaa = D-Phg K_i= 7.8 ± 0.5 pM	Yaa = Phg K_i=2.8 ± 0.4 pM Yaa = D-Phg K_i=0.095 ± 0.006 pM
	Xaa = Phe K_i=0.51 ± 0.05 pM Xaa = D-Phe K_i=3.4 ± 0.3 pM	Yaa = Phe K_i=4.5 ± 0.4 pM Yaa = D-Phe K_i=0.13 ± 0.05 pM

	Xaa = Hph K_i=0.18 ± 0.05 pM Xaa = D-Hph K_i=2.8 ± 0.5 pM	Yaa = Hph K_i=7.2 ± 0.3 pM Yaa = D-Hph K_i=0.78 ± 0.05 pM
	Xaa = His K_i=0.91 ± 0.04 pM Xaa = D-His K_i=2.1 ± 0.3 pM	Yaa = His K_i=6.2 ± 0.5 pM Yaa = D-His K_i=1.4 ± 0.4 pM
	Xaa = Trp K_i=630 ± 30 pM Xaa = D-Trp K_i=820 ± 50 pM	Yaa = Trp K_i=9.8 ± 0.4 pM Yaa = D-Trp K_i=2.2 ± 0.5 pM
	Xaa = Thi K_i=0.051 ± 0.004 pM Xaa = D-Thi K_i=2.8 ± 0.4 pM	Yaa = Thi K_i=5.1 ± 0.5 pM Yaa = D-Thi K_i=1.2 ± 0.4 pM

The preferred inhibitors having a K_i value smaller than 1 pM in accordance with the present invention are:

1) Bbs-Arg-(D-Pip)-Gly-Gly-(D-Ala)-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH; (SEQ ID NO:3)

2) Bbs-Arg-(D-Pip)-αAbu-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH; (SEQ ID NO:3)

3) Bbs-Arg-(D-Pip)-Gly-Gly-(D-αAbu)-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH; (SEQ ID NO:3)

4) Bbs-Arg-(D-Pip)-Nva-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH; (SEQ ID NO:3)

5) Bbs-Arg-(D-Pip)-Gly-Gly-(D-Nva)-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH; (SEQ ID NO:3)

6) Bbs-Arg-(D-Pip)-Nle-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH; (SEQ ID NO:3)

7) Bbs-Arg-(D-Pip)-Gly-Gly-(D-Nle)-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH; (SEQ ID NO:3)

8) Bbs-Arg-(D-Pip)-Met-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH; (SEQ ID NO:3)

9) Bbs-Arg-(D-Pip)-Val-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH; (SEQ ID NO:3)

10) Bbs-Arg-(D-Pip)-Gly-Gly-(D-Val)-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

11) Bbs-Arg-(D-Pip)-Gly-Gly-(D-Tbg)-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

12) Bbs-Arg-(D-Pip)-Ile-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

13) Bbs-Arg-(D-Pip)-Gly-Gly-(D-Ile)-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

14) Bbs-Arg-(D-Pip)-Leu-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

15) Bbs-Arg-(D-Pip)-Gly-Gly-(D-Leu)-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

16) Bbs-Arg-(D-Pip)-Chg-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

17) Bbs-Arg-(D-Pip)-Cha-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

18) Bbs-Arg-(D-Pip)-Gly-Gly-(D-Phg)-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

19) Bbs-Arg-(D-Pip)-Phe-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

20) Bbs-Arg-(D-Pip)-Gly-Gly-(D-Phe)-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

21) Bbs-Arg-(D-Pip)-Hph-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

22) Bbs-Arg-(D-Pip)-Gly-Gly-(D-Hph)-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

23) Bbs-Arg-(D-Pip)-His-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

and

24) Bbs-Arg-(D-Pip)-Thi-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH

(SEQ ID NO:3).

The more preferred inhibitors having a K_i value smaller than 0.1 pM in accordance with the present invention are:

1) Bbs-Arg-(D-Pip)-Nle-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

2) Bbs-Arg-(D-Pip)-Gly-Gly-(D-Phg)-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH;

(SEQ ID NO:3)

and

3) Bbs-Arg-(D-Pip)-Thi-Gly-Gly-Gly-βAla-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH

(SEQ ID NO:3).

It should be noted that a person skilled in the art could substitute suitable active site blocking segment, S' subsite blocking segment and fibrinogen recognition exosite blocking segment, and synthesize variants of such active trivalent hirudin-like inhibitors.

The inhibitors of the present invention have a higher affinity than the inhibitors of the prior art. This therefore represents a concrete benefit in the field of thrombin inhibitors.

While it may be possible that, for use in therapy, a compound of the invention may be administered as the raw chemical, it is preferable to present the active ingredient as a pharmaceutical formulation.

It will be appreciated by those skilled in the art that the compounds of formula (I) may be modified to provide pharmaceutically acceptable salts thereof which are included within the scope of the invention.

Pharmaceutically acceptable salts of the compounds of formula (I) include those derived from pharmaceutically acceptable inorganic and organic acids and bases. Examples of suitable acids include hydrochloric, hydrobromic, sulphuric, nitric, perchloric, fumaric, maleic, phosphoric, glycollic, lactic, salicylic, succinic, toluene-p-sulphonic, tartaric, acetic, citric, methanesulphonic, formic, benzoic, malonic, naphthalene-2-sulphonic and benzenesulphonic acids. Other acids such as oxalic, while not in themselves pharmaceutically acceptable, may be useful as intermediates in obtaining the

compounds of the invention and their pharmaceutically acceptable acid addition salts.

The invention thus further provides a pharmaceutical formulation comprising a compound of formula (I) and pharmaceutically acceptable acid addition salt thereof together with one or more pharmaceutically acceptable carriers therefor and, optionally, other therapeutic and/or prophylactic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

In a further embodiment of the present invention is provided the use of a compounds of formula (I) or a pharmaceutically acceptable salt in the manufacture of a medicament for the treatment of vascular disease in a mammal including human.

In an alternative aspect of the present invention is provided a method for the treatment of vascular disease for the treatment of a mammal, including human comprising the administration of an effective amount of a compound of formula (I).

It will be appreciated by people skilled in the art that treatment extends to prophylaxis as well to the treatment of established vascular disease.

The compounds of the present invention are useful in combinations, formulations and methods for the treatment and prophylaxis of vascular diseases. These diseases include myocardial infarction, stroke, pulmonary embolism, deep vein thrombosis, peripheral arterial occlusion, restenosis following arterial injury or invasive cardiological procedures, acute or chronic atherosclerosis, edema and inflammation, cancer and metastasis.

The term "combination" as used herein, includes a single dosage form containing at least one compound of this invention and at least one thrombolytic agent, a multiple dosage form, wherein the thrombin inhibitor and the thrombolytic agent are administered separately, but concurrently, or a multiple dosage form wherein the two components are administered separately, but sequentially. In sequential administration, the thrombin inhibitor may be given to the patient during the time period ranging from about 5 hours prior to about 5 hours after administration of the thrombolytic agent. Preferably, the thrombin inhibitor is administered to the patient during the period ranging from 2 hours prior to 2 hours following administration of the thrombolytic agent.

In these combinations, the thrombin inhibitor and the thrombolytic agent work in a complementary fashion to dissolve blood clots, resulting in decreased reperfusion times and increased reocclusion times in patients treated with them. Specifically, the thrombolytic agent dissolves the clot, while the thrombin inhibitor prevents newly exposed, clot-entrapped or clot-bound thrombin from regenerating the clot. The use of the thrombin inhibitor in the formulations of this invention advantageously allows the administration of a thrombolytic reagent in dosages previously considered too low to result in thrombolytic effects if given alone. This avoids some of the undesirable side effects associated with the use of thrombolytic agents, such as bleeding complications.

Thrombolytic agents which may be employed in the combinations of the present invention are those known in the art. Such agents include, but are not limited to, tissue plasminogen activator purified from natural sources, recombinant tissue plasminogen

activator, streptokinase, urokinase, purokinase, anisolated streptokinase plasminogen activator complex (ASPAC), animal salivary gland plasminogen activators and known, biologically active derivatives of any of the above.

Various dosage forms may be employed to administer the formulations and combinations of this invention. These include, but are not limited to, parenteral administration, oral administration and topical application. The formulations and combinations of this invention may be administered to the patient in any pharmaceutically acceptable dosage form, including those which may be administered to a patient intravenously as bolus or by continued infusion, intramuscularly -- including paravertebrally and periarticularly -- subcutaneously, intracutaneously, intra-articularly, intrasynovially, intrathecally, intra-lesionally, periostally or by oral, nasal, or topical routes. Such compositions and combinations are preferably adapted for topical, nasal, oral and parenteral administration, but, most preferably, are formulated for parenteral administration.

Parenteral compositions are most preferably administered intravenously either in a bolus form or as a constant infusion. For parenteral administration, fluid unit dose forms are prepared which contain the compounds of the present invention and a sterile vehicle. The compounds of this invention may be either suspended or dissolved, depending on the nature of the vehicle and the nature of the particular compounds of this invention. Parenteral compositions are normally prepared by dissolving the compounds of this invention in a vehicle, optionally together with other components, and filter sterilizing before filling into a suitable vial or ampule and sealing. Preferably,

adjuvants such as a local anesthetic, preservatives and buffering agents are also dissolved in the vehicle. The composition may then be frozen and lyophilized to enhance stability.

Parenteral suspensions are prepared in substantially the same manner, except that the active component is suspended rather than dissolved in the vehicle. Sterilization of the compositions is preferably achieved by exposure to ethylene oxide before suspension in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of its components.

Tablets and capsules for oral administration may contain conventional excipients, such as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, and wetting agents. The tablet may be coated according to methods well known in the art. Suitable fillers which may be employed include cellulose, mannitol, lactose and other similar agents. Suitable disintegrants include, but are not limited to, starch, polyvinylpyrrolidone and starch derivatives, such as sodium starch glycolate. Suitable lubricants include, for example, magnesium stearate. Suitable wetting agents include sodium lauryl sulfate.

Oral liquid preparations may be in the form of aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or another suitable vehicle before use. Such liquid preparations may contain conventional additives. These include suspending agents, such as sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethylcellulose, aluminum stearate gel or hydrogenated edible fats, emulsifying agents which

include lecithin, sorbitan monooleate, polyethylene glycols, or acacia, non-aqueous vehicles, such as almond oil, fractionated coconut oil, and oily esters, and preservatives, such as methyl or propyl p-hydroxybenzoate or sorbic acid.

Formulations for topical administration may, for example, be in aqueous jelly, oily suspension or emulsified ointment form.

The dosage and dose rate of the compounds of this invention will depend on a variety of factors, such as the weight of the patient, the specific pharmaceutical composition used, the object of the treatment, i.e., therapy or prophylaxis, the nature of the thrombotic disease to be treated, and the judgment of the treating physician.

According to the present invention, a preferred pharmaceutically effective daily dose of the compounds of this invention is between about 1 µg/kg body weight of the patient to be treated ("body weight") and about 5 mg/kg body weight. In combinations containing a thrombolytic agent, a pharmaceutically effective daily dose of the thrombolytic is between about 10% and 80% of the conventional dosage range. The "conventional dosage range" of a thrombolytic agent is the daily dosage used when that agent is employed in a monotherapy [physician's Desk Reference 1989, 43rd Edition, Edward R. Barnhart, publisher]. That conventional dosage range will, of course, vary depending on the thrombolytic agent employed. Examples of conventional dosage ranges are as follows: urokinase - 500,000 to 6,250,000 units/patient, streptokinase - 140,000 to 2,500,000 units/patient, tPA - 0.5 to 5.0 mg/kg body weight, ASPAC - 0.1 to 10 units/kg body weight.

Most preferably, the therapeutic and prophylactic compositions of the present invention comprise a dosage of between about 10 μg/kg body weight and about 500 μg/kg body weight of the compounds of this invention. Most preferred combinations comprise the same amount of the compounds of this invention and between about 10% and about 70% of the conventional dosage range of a thrombolytic agent. It should also be understood that a daily pharmaceutically effective dose of either the compounds of this invention or the thrombolytic agent present in combinations of the invention, may be less than or greater than the specific ranges cited above.

Once improvement in the patient's condition has occurred, a maintenance dose of a combination or composition of this invention is administered, if necessary. Subsequently, the dosage or the frequency of administration, or both, may be reduced, as a function of the symptoms, to a level at which the improved condition is retained. When the symptoms have been alleviated to the desired level, treatment should cease. Patients may, however, require intermittent treatment upon any recurrence of disease symptoms.

According to an alternate embodiment of this invention, compounds may be used in compositions and methods for coating the surfaces of invasive devices, resulting in a lower risk of clot formation or platelet activation in patients receiving such devices. Surfaces that may be coated with the compositions of this invention include, for example, prostheses, artificial valves, vascular grafts, stents and catheters. Methods and compositions for coating these devices are known to those of skill in the art. These include chemical cross-linking or physical adsorption of the compounds of this invention-containing

compositions to the surfaces of the devices. According to a further embodiment of the present invention, compounds may be used for *ex vivo* thrombus imaging in a patient. In this embodiment, the compounds of this invention are labeled with a radioisotope. The choice of radioisotope is based upon a number of well-known factors, for example, toxicity, biological half-life and detectability. Preferred radioisotopes include, but are not limited to ^{125}I, ^{123}I and ^{111}I. Techniques for labeling the compounds of this invention are well known in the art. Most preferably, the radioisotope is 123I and the labeling is achieved using 123I-Bolton-Hunter Reagent. The labeled thrombin inhibitor is administered to a patient and allowed to bind to the thrombin contained in a clot. The clot is then observed by utilizing well-known detecting means, such as a camera capable of detecting radioactivity coupled to a computer imaging system. This technique also yields images of platelet-bound thrombin and meizothrombin.

This invention also relates to compositions containing the compounds of this invention and methods for using such compositions in the treatment of tumor metastases. The efficacy of the compounds of this invention for the treatment of tumor metastases is manifested by the inhibition inhibitors to inhibit thrombin-induced endothelial cell activation. This inhibition includes the repression of platelet activation factor (PAF) synthesis by endothelial cells. These compositions and methods have important applications in the treatment of diseases characterized by thrombin-induced inflammation and edema, which is thought to be mediated be PAF. Such diseases include, but are not limited to, adult respiratory distress syndrome, septic shock, septicemia and reperfusion

damage. Early stages of septic shock include discrete, acute inflammatory and coagulopathic responses. It has previously been shown that injection of baboons with a lethal dose of live E. coli leads to marked declines in neutrophil count, blood pressure and hematocrit. Changes in blood pressure and hematocrit are due in part to the generation of a disseminated intravascular coagulopathy (DIC) and have been shown to parallel consumption of fibrinogen (F. B. Taylor et al., *J. Clin. Invest.*, **79**, pp. 918-25, 1987). Neutropenia is due to the severe inflammatory response caused by septic shock which results in marked increases in tumor necrosis factor levels. The compounds of this invention may be utilized in compositions and methods for treating or preventing DIC in septicemia and other diseases.

This invention also relates to the use of the above-described compounds, or compositions comprising them, as anticoagulants for extracorporeal blood. As used herein, the term "extracorporeal blood" includes blood removed in line from a patient, subjected to extracorporeal treatment, and then returned to the patient in such processes as dialysis procedures, blood filtration, or blood bypass during surgery. The term also includes blood products which are stored extracorporeally for eventual administration to a patient and blood collected from a patient to be used for various assays. Such products include whole blood, plasma, or any blood fraction in which inhibition of coagulation is desired.

The amount or concentration of compounds of this invention in these types of compositions is based on the volume of blood to be treated or, more preferably, its thrombin content. Preferably, an effective amount of a compounds of this invention of

this invention for preventing coagulation in extracorporeal blood is from about 1 μg/60 ml of extracorporeal blood to about 5 mg/60 ml of extracorporeal blood.

The compounds of this invention may also be used to inhibit clot-bound thrombin, which is believed to contribute to clot accretion. This is particularly important because commonly used anti-thrombin agents, such as heparin and low molecular weight heparin, are ineffective against clot-bound thrombin. Finally, the compounds of this invention may be employed in compositions and methods for treating neurodegenerative diseases. Thrombin is known to cause neurite retraction, a process suggestive of the rounding in shape changes of brain cells and implicated in neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I
EXPERIMENTAL PROCEDURES

Materials

Human α-thrombin (3000 NIH units/mg), Tos-Gly-Pro-Arg-AMC·HCl salt, poly(ethylene glycol) 8000, and Tris were purchased from Sigma. All Fmoc-amino acids, and all other amino acid derivatives for peptide synthesis were purchased from Advanced ChemTech, Bachem Bioscience Inc. and Calbiochem-Novabiochem. Fmoc-D-Glu(OtBu)-Wang resin (0.59 mmol/g) was purchased from Calbiochem-Novabiochem. The solvents for peptide synthesis were obtained from B&J Chemicals and Applied

Biosystems Inc. Trifluoroacetic acid was purchased from Halocarbon Products Co.

Peptide synthesis

The peptides were synthesized by the solid-phase method on a 396 Multiple Peptide Synthesizer (Advanced ChemTech) using a conventional Fmoc procedure. Peptides were cleaved from the resin using Reagent K (TFA 82.5%/water 5%/phenol 5%/thioanisole 5%/ethanedithiol 2.5%; 25 mL/g of peptide-resin) for 2-4 hours at room temperature. After precipitation with diethyl ether, peptides were filtered, dissolved in 50% acetic acid, and lyophilized. The peptides were then purified by a preparative HPLC (Vydac C_4 column, 4.6 X 25 cm) using a linear gradient of 20 to 50% acetonitrile in 0.1% TFA (0.5%/min gradient, 33 mL/min flow rate). The final products were lyophilized with 98% or higher purity estimated by an analytical HPLC (Vydac C_{18}, 0.46 X 25 cm column, 10-60% acetonitrile in 0.1% TFA, 1.0%/min gradient, 1.0 mL/min flow rate). The elution profile was monitored by an absorbance at 210 and 254 nm for the analytical HPLC, and 220 nm for the preparative HPLC. The peptides were identified with a Beckmann model 6300 amino acid analyzer and a SCIEX API III mass spectrometer. Amino acid analysis was used for peptide content determination. All peptides used in this article have correct amino acid composition and molecular mass.

Amidolytic assays

The inhibition of amidolytic activity of human α-thrombin was measured fluorometrically using Tos-Gly-Pro-Arg-AMC as a fluorogenic substrate in 50 mM Tris·HCl buffer (pH 7.80 at 25°C) containing 0.1 M NaCl and 0.1% poly(ethylene glycol) 8000 at room temperature (Szewczuk Z. et al. *Biochemistry* 31: 9132-9140, 1992). The final concentration of the inhibitors, the

substrate and human α-thrombin were 0.5-1000-fold of K_i, (1-8) X 10^{-6} M, and 3.0 X 10^{-11} M, respectively, if $K_i > 10^{-10}$ M; 10-100-fold of K_i, (5-40) X 10^{-6} M, and 3.0 X 10^{-11} M, respectively, if 10^{-10} M > K_i > 10^{-11} M; and (2-60) X 10^{-10} M, (5-40) X 10^{-6} M, and 3.0 X 10^{-11} M, respectively, if $K_i < 10^{-11}$ M. The hydrolysis of the substrate by thrombin was monitored on a Perkin Elmer LS50B luminescence spectrometer (λ_{ex}=383 nm; λ_{em}=455 nm) or on a Hitachi F2000 fluorescence spectrophotometer (λ_{ex}=383 nm; λ_{em}=455 nm), and the fluorescent intensity was calibrated using AMC. The substrate and an inhibitor were pre-mixed at appropriate concentrations (the solution volume was adjusted to 2.99 mL) before adding 10 μL of human α-thrombin (9 X 10^{-9} M). The reaction reached a steady state within 3 min after the hydrolysis started. The steady-state velocity was then measured for a few minutes. The kinetic data of the steay-state velocity at various concentrations of the substrate and the inhibitors of the competitive inhibition were analyzed using the methods described by Segal (Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems pp 100-160, John Wiley & Sons, 1975) and Szewczuk et al. (*Biochemistry*, 32: 3396-3404, 1993). A nonlinear regression program (Microsoft Excel) was used to estimate the kinetic parameters (K_m, V_{max}, and K_i). Some inhibitors with high affinity to thrombin showed a biphasic progress curves of the fluorescence change by time, which is typical phenomena of slow-tight binding inhibition (Morrison & Walsh, *Adv. in Enzymol.* 61: 201-301, 1988). The progress curves of the slow-tight binding inhibition were analyzed by the methods described by Stone & Hofsteenge (*Biochemistry* 25: 4622-4628, 1986).

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.